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(57) Abstract

The present invention relates to polynucleotide and polypeptide molecules for mammalian alpha helix-12 (Zalpha12). The polypeptides, and polynucleotides encoding them, are hormonal and may be used to regulate the functioning of the immune system. The present invention also includes antibodies to the Zalpha12 polypeptides. Antagonists may be used to treat inflammation and inflammation-related diseases.

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MAMMALIAN ALPHA-HELICAL PROTEIN - 12

BACKGROUND OF THE INVENTION

Inflammation normally is a localized, protective response to trauma or microbial invasion that destroys, dilutes, or walls-off the injurious agent and the injured tissue. It is characterized in the acute form by the classic signs of pain, heat, redness, swelling, and loss of function. Microscopically it involves a complex series of events, including dilation of arterioles, capillaries, and venules, with increased permeability and blood flow, exudation of fluids, including plasma proteins, and leukocyte migration into the area of inflammation.

Diseases characterized by inflammation are significant causes of morbidity and mortality in humans. Commonly, inflammation occurs as a defensive response to invasion of the host by foreign, particularly microbial, material. Responses to mechanical trauma, toxins, and neoplasia also may results in inflammatory reactions. The accumulation and subsequent activation of leukocytes are central events in the pathogenesis of most forms of inflammation. Deficiencies of inflammation compromise the host. Excessive inflammation caused by abnormal recognition of host tissue as foreign or prolongation of the inflammatory process may lead to inflammatory diseases as diverse as diabetes, arteriosclerosis, cataracts, reperfusion injury, and cancer, to post-infectious syndromes such as in infectious meningitis, rheumatic fever, and to rheumatic diseases such as systemic lupus erythematosus and rheumatoid arthritis. The centrality of the inflammatory response in these varied disease processes makes its regulation a major element in the prevention control or cure of human disease. Thus, there

is a need to discover cytokines which contribute to inflammation and inflammatory related diseases so that antagonists such as antibodies can be administered to down-regulate the cytokine so as to ameliorate the inflammation.

SUMMARY OF THE INVENTION

The present invention addresses this need by providing novel polypeptides and related compositions and methods and their antagonists. Within one aspect, the present invention provides an isolated polynucleotide encoding a mammalian cytokine termed Zalpha12. The data show that the cytokine is involved in the inflammation response. Thus, antagonists of Zalpha12 can be used to lessen inflammation especially inflammation associated with coronary heart disease, arteriosclerosis, Crohn's disease and inflammatory bowel disease.

Three variants have been discovered as shown in SEQ ID NOs:1, 2, 3, 4, 5 and 6. Each Zalpha12 polypeptide has a signal sequence which extends from amino acid residue 1, a methionine to and including amino acid residue 34, a serine of SEQ ID NOs:2, 4 and 6. Thus, the Zalpha12 polypeptide represented by SEQ ID NO:2 has a mature sequence extending from amino acid residue 35, an alanine, to and including amino acid residue 202,an asparagine, also represented by SEQ ID NO:8. The Zalpha12 polypeptide represented by SEQ ID NO:4 has a mature sequence extending from amino acid residue 35, an alanine to and including amino acid residue 288, an asparagine also represented by SEQ ID NO:9. The Zalpha12 polypeptide represented by SEQ ID NO:6 has a mature sequence extending from amino acid residue 35, an alanine to and including amino acid residue 158,an asparagine, also represented by SEQ ID NO:10.

Within a second aspect of the invention there is provided an expression vector comprising (a) a transcription promoter; (b) a DNA segment encoding Zalpha12 polypeptide, and (c) a transcription terminator, wherein the promoter, DNA segment, and terminator are operably linked.

Within a third aspect of the invention there is provided a cultured eukaryotic or prokaryotic cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses a protein polypeptide encoded by the DNA segment.

Within a further aspect of the invention there is provided a chimeric polypeptide consisting essentially of a first portion and a second portion joined by a peptide bond. The first portion of the chimeric polypeptide consists essentially of (a) a Zalpha12 polypeptide (b) allelic variants of Zalpha12; and (c) protein polypeptides that are at least 80% identical to (a) or (b). The second portion of the chimeric polypeptide consists essentially of another polypeptide such as an affinity tag. Within one embodiment the affinity tag is an immunoglobulin F_C polypeptide. The invention also provides expression vectors encoding the chimeric polypeptides and host cells transfected to produce the chimeric polypeptides.

Within an additional aspect of the invention there is provided an antibody that specifically binds to a Zalpha12 polypeptide as disclosed above, and also an anti-idiotypic antibody which neutralizes the antibody to a Zalpha12 polypeptide.

An additional embodiment of the present invention relates to a peptide or polypeptide which has the amino acid sequence of an epitope-bearing portion of a Zalpha12 polypeptide having an amino acid sequence described above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a Zalpha12

polypeptide of the present invention include portions of such polypeptides with at least nine, preferably at least 15 and more preferably at least 30 to 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the present invention described above are also included in the present invention. Examples of such epitope binding regions are SEQ ID NOs: 18, 19, 20, 25, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39, 40, 41, 42, 43 and 44. Also claimed are any of these polypeptides that are fused to another polypeptide or carrier molecule.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The teachings of all the references cited herein are incorporated in their entirety herein by reference.

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A, Nilsson *et al.*, *EMBO J.* *4*:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* *198*:3 (1991), glutathione S transferase, Smith and Johnson, *Gene* *67*:31 (1988), Glu-Glu affinity tag, Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* *82*:7952-4 (1985), substance P, Flag™ peptide, Hopp *et al.*, *Biotechnology* *6*:1204-1210 (1988), streptavidin binding

peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification 2*: 95-107 (1991). DNAs encoding affinity tags are available from commercial suppliers (*e.g.*, Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the

complement/anti-complement pair preferably has a binding affinity of $<10^9\ M^{-1}$.

The term "complements of a polynucleotide molecule" is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a polynucleotide that has a contiguous stretch of identical or complementary sequence to another polynucleotide. Contiguous sequences are said to "overlap" a given stretch of polynucleotide sequence either in their entirety or along a partial stretch of the polynucleotide. For example, representative contigs to the polynucleotide sequence 5'-ATGGCTTAGCTT-3' are 5'-TAGCTTgagtct-3' and 3'-gtcgacTACCGA-5'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free

of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* *316*:774-78 (1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, *e.g.*, transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, a-globin, b-globin, and myoglobin are paralogs of each other.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-

peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating.hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (*e.g.*, gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

Introduction

The Zalpha12 protein is an alpha helical protein. SEQ ID NO:2 has four alpha helices, A, B, C and D. Helix A of SEQ ID NO:2 extends from amino acid residue 56, a leucine, to and including amino acid residue 70, an isoleucine also defined by SEQ ID NO:21. Helix B of SEQ ID NO:2 extends from amino acid residue 96, a leucine to and including amino acid residue 110, a tyrosine, also defined by SEQ ID NO:22. Helix C of SEQ ID NO:2 extends from amino acid residue 130, a leucine, to and including amino acid residue 144, a leucine, also defined by SEQ ID NO:23. Helix D of SEQ ID NO: 2 extends from amino acid residue 162, a methionine, to and including amino acid residue 176, a serine, also defined by SEQ ID NO:24. SEQ ID NO:25 contains helices A + B; SEQ ID NO:26 contains helices A + B + C; SEQ ID NO:27 contains helices A + B + C + D; SEQ ID NO:28 contains helices B + C + D; SEQ ID NO:29 contains helices B + C; and SEQ ID NO:30 contains helices C + D of SEQ ID NO:2.

The polypeptide of SEQ ID NO:4 also contains four helices A, B, C and D. Helix A of SEQ ID NO:4 extends from amino acid residue 45, a histidine, to and including amino acid residue 59, a leucine, also defined by SEQ ID NO:31. Helix B of SEQ ID NO:4 extends from amino acid residue 116, a valine, to and including amino acid residue 130, a lysine, also defined by SEQ ID NO:32. Helix C of SEQ ID NO:4 extends from amino acid residue 142, a leucine, to and including amino acid residue 156, an isoleucine, also defined by SEQ ID NO:33. Helix D of SEQ ID NO:4 extends from amino acid residue 182, a leucine, to and including amino acid residue 196, a tyrosine, also defined by SEQ ID NO:34. SEQ ID NO:35 contains helices A + B of SEQ ID NO:4. SEQ ID NO:36 contains helices A + B + C of SEQ ID NO:4. SEQ ID NO:37 contains helices A + B + C + D of SEQ ID NO:4. SEQ ID NO: 38 contains helices B + C + D of SEQ ID NO:4. SEQ ID NO:39 contains helices B + C of SEQ ID NO:4. SEQ ID NO:40 contains helices C + D of SEQ ID NO:4.

Expression of the *Zalpha12* gene is seen in a number of different tissues including the spleen, thymus, testis, small intestines, colon, peripheral blood lymphocytes (PBL), stomach, trachea, T-cells including CD4+ and CD8+ cells, and bone marrow. This pattern of expression suggests that zalpha12 may play a general role in development and exert important regulatory control of testicular differentiation, of the hypothalamic-pituitary-gonadal axis, and of gonadal steroidogenesis and spermatogenesis.

Development of testicular hormone production can be divided into early and late steps, with the latter dependent on the activation of functionally-determined Leydig cell precursors by luteinizing hormone (LH). However, the factors that control the early steps in this process remain unknown, Huhtaniemi , *Reprod. Fertil. Dev.* *7:* 1025-1035 (1995) suggesting that zalpha12 might be

responsible for activation of a non-steroidogenic, non-LH responsive precursor cell.

Once Leydig cell differentiation has occurred, production of steroid hormones in the testis is dependent on the secretion of the gonadotrophins, LH and follicle-stimulating hormone (FSH), by the pituitary. LH stimulates production of testosterone by the Leydig cells, whereas spermatogenesis depends on both FSH and high intratesticular testosterone concentrations. LH and FSH secretion is in turn under control of gonadotrophin releasing hormone (GnRH) produced in the hypothalamus, Kaufman, *The neuro endocrine regulation of male reproduction*. in: Male Infertility. Clinical Investigation, Cause Evaluation and Treatment., FH Comhaire, ed., pp 29-54(Chapman and Hall, London, 1996). Since testicular products have been shown to control LH and FSH production, this suggests that zalpha12 might regulate hormone production by the hypothalamus.

It is well known that steroidogenesis and spermatogenesis take place within two different cellular compartments of the testes, with Leydig and Sertoli cells responsible for the former and latter, respectively, Saez , *Endocrin. Rev. 15*: 574-626 (1994). The activity of each of these cell types appears to be regulated by the secretory products of the other. Sertoli cell derived tumor necrosis factor-a, fibroblast growth factor, interleukin-1, transforming growth factor-B, epidermal growth factor/transforming growth factor-a, activin, inhibin, insulin-like growth factor-1, platelet derived growth factor, endothelin, and ariginine-vasopressin have all been shown to regulate Leydig cell function, Saez , *Endocrin. Rev. 15:* 574-626 (1994). Thus, zalpha12 might control or modulate the activities of one or more of these genes.

In men, aging is associated with a progressive decline in testicular function. These changes are

manifest clinically by decreased virility, vigor, and libido that point towards a relative testicular deficiency, Vermeulen, *Ann. Med.* 25:531-534 (1993); Pugeat et al., *Horm. Res.* 43: 104-110 (1995). Hormone replacement therapy in elderly men is not currently recommended which suggests that a new therapy for the male climacterium would be very valuable.

POLYNUCLEOTIDES:

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the Zalpha12 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules.

Polynucleotides, generally a cDNA sequence, of the present invention encode the described polypeptides herein. A cDNA sequence which encodes a polypeptide of the present invention is comprised of a series of codons, each amino acid residue of the polypeptide being encoded by a codon and each codon being comprised of three nucleotides. The amino acid residues are encoded by their respective codons as follows.

Alanine (Ala) is encoded by GCA, GCC, GCG or GCT;

Cysteine (Cys) is encoded by TGC or TGT;

Aspartic acid (Asp) is encoded by GAC or GAT;

Glutamic acid (Glu) is encoded by GAA or GAG;

Phenylalanine (Phe) is encoded by TTC or TTT;

Glycine (Gly) is encoded by GGA, GGC, GGG or GGT;

Histidine (His) is encoded by CAC or CAT;

Isoleucine (Ile) is encoded by ATA, ATC or ATT;

Lysine (Lys) is encoded by AAA, or AAG;

Leucine (Leu) is encoded by TTA, TTG, CTA, CTC, CTG or CTT;

Methionine (Met) is encoded by ATG;

Asparagine (Asn) is encoded by AAC or AAT;

Proline (Pro) is encoded by CCA, CCC, CCG or CCT;

Glutamine (Gln) is encoded by CAA or CAG;

Arginine (Arg) is encoded by AGA, AGG, CGA, CGC, CGG or CGT;

Serine (Ser) is encoded by AGC, AGT, TCA, TCC, TCG or TCT;

Threonine (Thr) is encoded by ACA, ACC, ACG or ACT;

Valine (Val) is encoded by GTA, GTC, GTG or GTT;

Tryptophan (Trp) is encoded by TGG; and

Tyrosine (Tyr) is encoded by TAC or TAT.

It is to be recognized that according to the present invention, when a polynucleotide is claimed as described herein, it is understood that what is claimed are both the sense strand, the anti-sense strand, and the DNA as double-stranded having both the sense and anti-sense strand annealed together by their respective hydrogen bonds. Also claimed is the messenger RNA (mRNA) which encodes the polypeptides of the president invention, and which mRNA is encoded by the cDNA described herein. Messenger RNA (mRNA) will encode a polypeptide using the same codons as those defined herein, with the exception that each thymine nucleotide (T) is replaced by a uracil nucleotide (U).

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, *et al.*, *Nuc. Acids Res.* *8*:1893-1912 (1980); Haas, *et al.* *Curr. Biol.* *6*:315-324 (1996); Wain-Hobson, *et al.*, *Gene*

13:355-364 (1981); Grosjean and Fiers, *Gene 18*:199-209 (1982); Holm, *Nuc. Acids Res. 14*:3075-3087 (1986); Ikemura, *J. Mol. Biol. 158*:573-597 (1982). As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid Threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NOs:1, 3 or 5 or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is up to about 0.03 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for preparing DNA and RNA are well known in the art. In general, RNA is isolated from a tissue or cell that produces large amounts of Zalpha12 RNA. Such tissues and cells are identified by Northern blotting, Thomas, *Proc. Natl. Acad. Sci. USA* 77:5201 (1980) and are discussed below. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient, Chirgwin *et al.*, *Biochemistry 18*:52-94 (1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder, *Proc. Natl. Acad. Sci. USA 69*:1408-1412 (1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. In the alternative, genomic DNA can be isolated. Polynucleotides encoding Zalpha12 polypeptides are then identified and isolated by, for example, hybridization or PCR.

A full-length clone encoding Zalpha12 can be obtained by conventional cloning procedures. Complementary DNA (cDNA) clones are preferred, although for some applications (*e.g.*, expression in transgenic animals) it may be preferable to use a genomic clone, or to modify a cDNA clone to include at least one genomic intron. Methods for preparing cDNA and genomic clones are well known and within the level of ordinary skill in the art, and include the use of the sequence disclosed herein, or parts thereof, for probing or priming a library. Expression libraries can be probed with antibodies to Zalpha12, receptor fragments, or other specific binding partners.

The polynucleotides of the present invention can also be synthesized using DNA synthesizers. Currently the method of choice is the phosphoramidite method. If chemically synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made

separately. The production of short genes (60 to 80 bp) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes (>300 bp), however, special strategies must be invoked, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length.

See Glick and Pasternak, *Molecular Biotechnology, Principles & Applications of Recombinant DNA,* (ASM Press, Washington, D.C. 1994); Itakura *et al.*, *Annu. Rev. Biochem.* *53*: 323-356 (1984) and Climie *et al.*, *Proc. Natl. Acad. Sci. USA* *87*:633-637 (1990).

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zalpha12 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zalpha12 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses Zalpha12 as disclosed herein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A Zalpha12-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also

be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the representative human Zalpha12 sequence disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zalpha12 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NOs:1, 3 or 5 represent specific alleles of human Zalpha12 and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures.. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOs:2, 4 or 6. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the Zalpha12 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention also provides isolated Zalpha12 polypeptides that are substantially identical to the polypeptides of SEQ ID NOs:2; 4, 6, 8, 9 or 10 and their orthologs. The term "substantially identical" is used herein to denote polypeptides having 50%, 60%, 70%, 80% and most preferably at least 90%, 95% or 99% sequence identity to the sequences shown in SEQ ID NOs:2, 4, 6, 8, 9 or 10 or their orthologs. Percent sequence identity is

determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* *48*: 603-616 (1986) and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* *89*:10915-10919 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM 62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 1 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$

Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Those skilled in the art appreciate that there are many established algorithms to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA 85*:2444 (1988), and by Pearson, *Meth. Enzymol. 183*:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions or deletions. The ten regions with the highest density of identities are then re-scored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol. 48*:444 (1970); Sellers, *SIAM J. Appl. Math. 26*:787 (1974), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by

modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol. 183*:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably form four to six.

The present invention includes nucleic acid molecules that encode a polypeptide having one or more conservative amino acid changes, compared with the amino acid sequence of SEQ ID NOs:2, 4, 6, 8, 9 or 10. The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins [Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA 89*:10915 (1992)]. Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. As used herein, the language "conservative amino acid substitution" refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0,1,2, or 3. Preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (*e.g.*, 1,2 or 3), while more preferred conservative substitutions are characterized by a BLOSUM62 value of at least 2 (*e.g.*, 2 or 3).

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Variant Zalpha12 polypeptides or substantially homologous Zalpha12 polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 2) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. The present invention thus includes polypeptides of from 20 to 30 amino acid residues that comprise a sequence that is at least 90%, preferably at least 95%, and more preferably 99% or more identical to the corresponding region of SEQ ID NOs:2, 4, 6, 8, 9 or 10. Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the Zalpha12 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine

Table 2 cont.

Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The present invention further provides a variety of other polypeptide fusions [and related multimeric proteins comprising one or more polypeptide fusions]. For example, a Zalpha12 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-Zalpha12 polypeptide fusions can be expressed in genetically engineered cells [to produce a variety of multimeric Zalpha12 analogs]. Auxiliary domains can be fused to Zalpha12 polypeptides to target them to specific cells, tissues, or macromolecules (*e.g.*, collagen). For example, a Zalpha12 polypeptide or protein could be targeted to a predetermined cell type by fusing a Zalpha12 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A Zalpha12 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between

domains. See, Tuan *et al.*, *Connective Tissue Research* *34*:1-9 (1996).

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs.

Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* *113*:2722 (1991); Ellman *et al.*, *Methods Enzymol.* *202*:301 (1991; Chung *et al.*, *Science* *259*:806-809 (1993); and Chung *et al.*, *Proc. Natl. Acad. Sci. USA* *90*:10145-1019 (1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs, Turcatti *et al.*, *J. Biol. Chem.* *271*:19991-19998 (1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino

acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* *33*:7470-7476 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions, Wynn and Richards, *Protein Sci.* *2*:395-403 (1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zalpha12 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis, Cunningham and Wells, *Science 244*: 1081-1085 (1989); Bass *et al.*, *Proc. Natl. Acad. Sci. USA 88*:4498-502 (1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, *J. Biol. Chem. 271*:4699-708, 1996. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, *Science 255*:306-312 (1992); Smith *et al.*, *J.*

Mol. Biol. 224:899-904 (1992); Wlodaver *et al.*, *FEBS Lett. 309*:59-64 (1992).

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer, *Science 241*:53-57 (1988) or Bowie and Sauer, *Proc. Natl. Acad. Sci. USA 86*:2152-2156 (1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display, *e.g.*, Lowman *et al.*, *Biochem. 30*:10832-10837 (1991); Ladner *et al.*, U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis, Derbyshire *et al.*, *Gene 46*:145 (1986); Ner *et al.*, *DNA* 7:127 (1988).

Variants of the disclosed Zalpha12 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, *Nature 370*:389-391, (1994), Stemmer, *Proc. Natl. Acad. Sci. USA 91*:10747-10751 (1994) and WIPO Publication WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods

to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed herein, one of ordinary skill in the art can identify and/or prepare a variety of polypeptide fragments or variants of SEQ ID NOs:2, 4 or 6 or that retain the properties of the wild-type Zalpha12 protein.

For any Zalpha12 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above.

PROTEIN PRODUCTION

The Zalpha12 polypeptides of the present invention, including full-length polypeptides, biologically active fragments, and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook *et al.*, Molecular Cloning: *A Laboratory Manual*, 2nd ed., (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and Ausubel *et al.*,

eds., *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc., NY, 1987).

In general, a DNA sequence encoding a Zalpha12 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a Zalpha12 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of Zalpha12, or may be derived from another secreted protein (*e.g.*, t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the Zalpha12 DNA sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the polypeptides of the present invention is

used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, Wigler *et al.*, *Cell* *14*:725 (1978); Corsaro and Pearson, *Somatic Cell Genetics* 7:603 (1981); Graham and Van der Eb, *Virology* *52*:456 (1973), electroporation, Neumann *et al.*, *EMBO J.* *1*:841-845 (1982), DEAE-dextran mediated transfection (Ausubel *et al.*, *ibid.*, and liposome-mediated transfection, Hawley-Nelson *et al.*, *Focus* *15*:73 (1993); Ciccarone *et al.*, *Focus* *15*:80 (1993), and viral vectors, Miller and Rosman, *BioTechniques* 7:980(1989); Wang and Finer, *Nature Med.* *2*:714 (1996). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson *et al.*, U.S. Patent No. 4,713,339; Hagen *et al.*, U.S. Patent No. 4,784,950; Palmiter *et al.*, U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham *et al.*, *J. Gen. Virol.* *36*:59 (1977) and Chinese hamster ovary (*e.g.* CHO-K1; ATCC No. CCL 61) cell

lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, *e.g.*, U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.* hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including plant cells, insect cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar *et al.*, *J. Biosci. (Bangalore) 11*:47 (1987). Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino *et al.*, U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from Autographa californica nuclear polyhedrosis virus (AcNPV). DNA encoding the Zalpha12 polypeptide is inserted into the baculoviral genome in place of the AcNPV polyhedrin gene coding sequence by one of two methods. The first is the traditional method of homologous DNA recombination between wild-type AcNPV and a transfer vector containing the Zalpha12 flanked by AcNPV sequences. Suitable insect cells, *e.g.* SF9 cells, are infected with wild-type AcNPV and transfected with a transfer vector comprising a Zalpha12 polynucleotide operably linked to an AcNPV polyhedrin gene promoter, terminator, and flanking sequences. See, King, L.A. and Possee, R.D., *The Baculovirus Expression System: A Laboratory Guide*, (Chapman & Hall, London); O'Reilly, D.R. *et al.*, *Baculovirus Expression Vectors: A Laboratory Manual* (Oxford University Press, New York, New York, 1994); and, Richardson, C. D., Ed., *Baculovirus Expression Protocols. Methods in Molecular Biology*, (Humana Press, Totowa, NJ 1995). Natural recombination within an insect cell will result in a recombinant baculovirus which contains Zalpha12 driven by the polyhedrin promoter. Recombinant viral stocks are made by methods commonly used in the art.

The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow, V.A, *et al.*, *J Virol 67*:4566 (1993). This

system is sold in the Bac-to-Bac kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zalpha12 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case Zalpha12. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as *Pcor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins, M.S. and Possee, R.D., *J Gen Virol 71*:971 (1990); Bonning, B.C. *et al.*, *J Gen Virol 75*:1551 (1994); and, Chazenbalk, G.D., and Rapoport, B., *J Biol Chem 270*:1543 (1995). In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native Zalpha12 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native Zalpha12 secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zalpha12 polypeptide, for example, a Glu-Glu epitope tag, Grussenmeyer, T. *et al.*, *Proc Natl Acad Sci. 82*:7952 (1985). Using a technique known in the art, a transfer vector containing Zalpha12 is transformed into *E. coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant

baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, *e.g.* Sf9 cells. Recombinant virus that expresses Zalpha12 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

The recombinant virus is used to infect host cells, typically a cell line derived from the fall army worm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, *Molecular Biotechnology: Principles and Applications of Recombinant DNA* (ASM Press, Washington, D.C., 1994). Another suitable cell line is the High FiveO™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent #5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cellO405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. The recombinant virus-infected cells typically produce the recombinant Zalpha12 polypeptide at 12-72 hours post-infection and secrete it with varying efficiency into the medium. The culture is usually harvested 48 hours post-infection. Centrifugation is used to separate the cells from the medium (supernatant). The supernatant containing the Zalpha12 polypeptide is filtered through micropore filters, usually 0.45 μm pore size. Procedures used are generally described in available laboratory manuals (King, L. A. and Possee, R.D., *ibid.*; O'Reilly, D.R. *et al.*, *ibid.*; Richardson, C. D., *ibid.*). Subsequent purification

of the Zalpha12 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki *et al.*, U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch *et al.*, U.S. Patent No. 5,037,743; and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311; Kingsman *et al.*, U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al.*, *J. Gen. Microbiol.* *132*:3459 (1986) and Cregg, U.S. Patent No. 4,882,279.

Aspergillus cells may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of

interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art, see, *e.g.*, Sambrook *et al.*, *ibid.*). When expressing a Zalpha12 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins

and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and 0.006% L-leucine).

Another embodiment of the present invention provides for a peptide or polypeptide comprising an epitope-bearing portion of a Zalpha12 polypeptide of the invention. The epitope of the this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. A region of a protein to which an antibody can bind is defined as an "antigenic epitope". See for instance, Geysen, H.M. *et al., Proc. Natl. Acad Sci. USA 81:*3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in the art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See Sutcliffe, J.G. *et al. Science 219:*660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined

neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer soluble peptides, especially those containing proline residues, usually are effective.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that react with the protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues and hydrophobic residues are preferably avoided); and sequences containing proline residues are particularly preferred. All of the polypeptides shown in the sequence listing contain antigenic epitopes to be used according to the present invention. The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a Zalpha12 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods [see, for example,

Geysen *et al.*, *supra*. See also U.S. Patent No. 4,708,781 (1987) further describes how to identify a peptide bearing an immunogenic epitope of a desired protein.

Protein Isolation

It is preferred to purify the polypeptides of the present invention to ≥80% purity, more preferably to ≥90% purity, even more preferably ≥95% purity, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Expressed recombinant Zalpha12 polypeptides (or chimeric Zalpha12 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which

they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988).

The polypeptides of the present invention can be isolated by exploitation of their properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate, Sulkowski, *Trends in Biochem.* *3*:1 (1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography. *Methods in Enzymol.*, Vol. *182*, "Guide to Protein Purification", M. Deutscher, (ed.),page 529-539 (Acad. Press, San Diego, 1990). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (*e.g.*,

maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Moreover, using methods described in the art, polypeptide fusions, or hybrid Zalpha12 proteins, are constructed using regions or domains of the inventive Zalpha12, Sambrook *et al.*, *ibid.*, Altschul *et al.*, *ibid.*, Picard, *Cur. Opin. Biology*, 5:511 (1994). These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure.

Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. For example, part or all of a domain(s) conferring a biological function may be swapped between Zalpha12 of the present invention with the functionally equivalent domain(s) from another family member. Such domains include, but are not limited to, the secretory signal sequence, conserved, and significant domains or regions in this family. Such fusion proteins would be expected to have a biological functional profile that is the same or similar to polypeptides of the present invention or other known family proteins, depending on the fusion constructed. Moreover, such fusion proteins may exhibit other properties as disclosed herein.

Zalpha12 polypeptides or fragments thereof may also be prepared through chemical synthesis. Zalpha12 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

Chemical Synthesis of Polypeptides

Polypeptides, especially polypeptides of the present invention can also be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis, for example as described by Merrifield, *J. Am. Chem. Soc. 85*:2149 (1963).

ASSAYS

The activity of molecules of the present invention can be measured using a variety of assays. Of particular interest are changes in steroidogenesis, spermatogenesis, in the testis, LH and FSH production and GnRH in the hypothalamus. Such assays are well known in the art.

Proteins of the present invention are useful for increasing sperm production. Zalpha12 can be measured *in vitro* using cultured cells or *in vivo* by administering molecules of the claimed invention to the appropriate animal model. For instance, Zalpha12 transfected (or co-transfected) expression host cells may be embedded in an alginate environment and injected (implanted) into recipient animals. Alginate-poly-L-lysine microencapsulation, permselective membrane encapsulation and diffusion chambers have been described as a means to entrap transfected mammalian cells or primary mammalian cells. These types of non-immunogenic "encapsulations" or microenvironments permit the transfer of nutrients into the microenvironment, and also permit the diffusion of proteins and other macromolecules secreted or released by the captured cells across the environmental barrier to the recipient animal. Most importantly, the capsules or microenvironments mask and shield the foreign, embedded cells from the recipient animal's immune response. Such

microenvironments can extend the life of the injected cells from a few hours or days (naked cells) to several weeks (embedded cells).

Alginate threads provide a simple and quick means for generating embedded cells. The materials needed to generate the alginate threads are readily available and relatively inexpensive. Once made, the alginate threads are relatively strong and durable, both *in vitro* and, based on data obtained using the threads, *in vivo*. The alginate threads are easily manipulable and the methodology is scalable for preparation of numerous threads. In an exemplary procedure, 3% alginate is prepared in sterile H_2O, and sterile filtered. Just prior to preparation of alginate threads, the alginate solution is again filtered. An approximately 50% cell suspension (containing about 5×10^5 to about 5×10^7 cells/ml) is mixed with the 3% alginate solution. One ml of the alginate/cell suspension is extruded into a 100 mM sterile filtered $CaCl_2$ solution over a time period of ~15 min, forming a "thread". The extruded thread is then transferred into a solution of 50 mM $CaCl_2$, and then into a solution of 25 mM $CaCl_2$. The thread is then rinsed with deionized water before coating the thread by incubating in a 0.01% solution of poly-L-lysine. Finally, the thread is rinsed with Lactated Ringer's Solution and drawn from solution into a syringe barrel (without needle attached). A large bore needle is then attached to the syringe, and the thread is intraperitoneally injected into a recipient in a minimal volume of the Lactated Ringer's Solution.

An alternative *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review,

see T.C. Becker *et al.*, *Meth. Cell Biol.* *43*:161 (1994); and J.T. Douglas and D.T. Curiel, *Science & Medicine* *4*:44 (1997). The adenovirus system offers several advantages: adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with a large number of available vectors containing different promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (the human 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (*e.g.*, liver) will express and process (and, if a secretory signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free

conditions, which allows infected cells to survive for several weeks without significant cell division. Alternatively, adenovirus vector infected 293S cells can be grown in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier *et al.*, *Cytotechnol.* *15*:145 (1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant. Within the infected 293S cell production protocol, non-secreted proteins may also be effectively obtained.

Agonists and Antagonists

In view of the tissue distribution observed for Zalpha12, agonists (including the natural ligand/ substrate/ cofactor/ etc.) and antagonists have enormous potential in both *in vitro* and *in vivo* applications. Compounds identified as Zalpha12 agonists are useful for stimulating the immune system or spermatogenesis. For example, Zalpha12 and agonist compounds are useful as components of defined cell culture media, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture.

Antagonists

Antagonists are also useful as research reagents for characterizing sites of ligand-receptor interaction. Antagonists of Zalpha12 can also be used to down-regulate inflammation as discussed in more further detail below. Inhibitors of Zalpha12 activity (Zalpha12 antagonists) include anti-Zalpha12 antibodies and soluble Zalpha12 receptors, as well as other peptidic and non-peptidic agents (including ribozymes).

Zalpha12 can also be used to identify inhibitors (antagonists) of its activity. Test compounds are added to the assays disclosed herein to identify compounds that inhibit the activity of Zalpha12. In addition to those assays disclosed herein, samples can be tested for

inhibition of Zalpha12 activity within a variety of assays designed to measure receptor binding or the stimulation/inhibition of Zalpha12-dependent cellular responses. For example, Zalpha12-responsive cell lines can be transfected with a reporter gene construct that is responsive to a Zalpha12-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a Zalpha12-DNA response element operably linked to a gene encoding a protein which can be assayed, such as luciferase. DNA response elements can include, but are not limited to, cyclic AMP response elements (CRE), hormone response elements (HRE) insulin response element (IRE), Nasrin *et al.*, *Proc. Natl. Acad. Sci. USA 87*:5273 (1990) and serum response elements (SRE) (Shaw *et al. Cell 56*: 563 (1989). Cyclic AMP response elements are reviewed in Roestler *et al.*, *J. Biol. Chem. 263* (19):9063 (1988) and Habener, *Molec. Endocrinol. 4* (8):1087 (1990). Hormone response elements are reviewed in Beato, *Cell 56*:335 (1989). Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of Zalpha12 on the target cells as evidenced by a decrease in Zalpha12 stimulation of reporter gene expression. Assays of this type will detect compounds that directly block Zalpha12 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or other samples can be tested for direct blocking of Zalpha12 binding to receptor using Zalpha12 tagged with a detectable label (*e.g.*, ^{125}I, biotin, horseradish peroxidase, FITC, and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled Zalpha12 to the receptor is indicative of inhibitory activity, which can be confirmed through secondary assays. Receptors used within binding assays

may be cellular receptors or isolated, immobilized receptors.

A Zalpha12 polypeptide can be expressed as a fusion with an immunoglobulin heavy chain constant region, typically an F_C fragment, which contains two constant region domains and lacks the variable region. Methods for preparing such fusions are disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Such fusions are typically secreted as multimeric molecules wherein the Fc portions are disulfide bonded to each other and two non-Ig polypeptides are arrayed in closed proximity to each other. Fusions of this type can be used to affinity purify the ligand. For use in assays, the chimeras are bound to a support via the F_C region and used in an ELISA format.

A Zalpha12 ligand-binding polypeptide can also be used for purification of ligand. The polypeptide is immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting medium will generally be configured in the form of a column, and fluids containing ligand are passed through the column one or more times to allow ligand to bind to the receptor polypeptide. The ligand is then eluted using changes in salt concentration, chaotropic agents (guanidine HCl), or pH to disrupt ligand-receptor binding.

An assay system that uses a ligand-binding receptor (or an antibody, one member of a complement/anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore, Pharmacia Biosensor, Piscataway, NJ) may be advantageously

employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of this instrument is disclosed by Karlsson, *J. Immunol. Methods* *145*:229 (1991) and Cunningham and Wells, *J. Mol. Biol.* *234*:554 (1993). A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

Ligand-binding receptor polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity, Scatchard, *Ann. NY Acad. Sci.* *51*: 660 (1949) and calorimetric assays, Cunningham *et al.*, *Science* *253*:545 (1991); Cunningham *et al.*, *Science* *245*:821 (1991).

Zalpha12 polypeptides can also be used to prepare antibodies that specifically bind to Zalpha12 epitopes, peptides or polypeptides. The Zalpha12 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal and elicit an immune response. Suitable antigens would be the Zalpha12 polypeptides encoded by SEQ ID NOs: 2, 4, 6, 8- 10, 18-20, 25-30 and 35-44. Antibodies generated from this immune response can be isolated and purified as described herein. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, *Current Protocols in Immunology*, Cooligan, *et al.*

(eds.), National Institutes of Health, (John Wiley and Sons, Inc., 1995); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, Second Edition* (Cold Spring Harbor, NY, 1989); and Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications* (CRC Press, Inc., Boca Raton, FL, 1982).

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a Zalpha12 polypeptide or a fragment thereof. The immunogenicity of a Zalpha12 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zalpha12 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as $F(ab')_2$, Fab proteolytic fragments, Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody).

In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to Zalpha12 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zalpha12 protein or peptide). Genes encoding polypeptides having potential Zalpha12 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner *et al.*, US Patent NO. 5,223,409; Ladner *et al.*, US Patent NO. 4,946,778; Ladner *et al.*, US Patent NO. 5,403,484 and Ladner *et al.*, US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zalpha12 sequences disclosed herein to identify proteins which bind to Zalpha12. These "binding proteins" which interact

with Zalpha12 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding proteins can also be used in analytical methods such as for screening expression libraries and neutralizing activity. The binding proteins can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease. These binding proteins can also act as Zalpha12 "antagonists" to block Zalpha12 binding and signal transduction *in vitro* and *in vivo*.

Antibodies are determined to be specifically binding if: (1) they exhibit a threshold level of binding activity, and (2) they do not significantly cross-react with related polypeptide molecules. First, antibodies herein specifically bind if they bind to a Zalpha12 polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis.

Second, antibodies are determined to specifically bind if they do not significantly cross-react with related polypeptides. Antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zalpha12 but not known related polypeptides using a standard Western blot analysis (Ausubel *et al.*, *ibid.*). Examples of known related polypeptides are orthologs, proteins from the same species that are members of a protein family (*e.g.* IL-16), Zalpha12 polypeptides, and non-human Zalpha12. Moreover, antibodies may be "screened against" known related

polypeptides to isolate a population that specifically binds to the inventive polypeptides. For example, antibodies raised to Zalpha12 are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to Zalpha12 will flow through the matrix under the proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to closely related polypeptides, *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.) (Cold Spring Harbor Laboratory Press, 1988); *Current Protocols in Immunology*, Cooligan, *et al.* (eds.), National Institutes of Health (John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, *Fundamental Immunology*, Paul (eds.) (Raven Press, 1993); Getzoff *et al.*, *Adv. in Immunol. 43*: 1-98 (1988); *Monoclonal Antibodies: Principles and Practice*, Goding, J.W. (eds.), (Academic Press Ltd., 1996); Benjamin *et al.*, *Ann. Rev. Immunol. 2*: 67-101 (1984).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to Zalpha12 proteins or peptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.) (Cold Spring Harbor Laboratory Press, 1988). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant Zalpha12 protein or polypeptide.

Antibodies to Zalpha12 may be used for tagging cells that express Zalpha12; for isolating Zalpha12 by affinity purification; for diagnostic assays for determining circulating levels of Zalpha12 polypeptides;

for detecting or quantitating soluble Zalpha12 as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block Zalpha12 *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to Zalpha12 or fragments thereof may be used *in vitro* to detect denatured Zalpha12 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

BIOACTIVE CONJUGATES:

Antibodies or polypeptides herein can also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. For instance, polypeptides or antibodies of the present invention can be used to identify or treat tissues or organs that express a corresponding anti-complementary molecule (receptor or antigen, respectively, for instance). More specifically, Zalpha12 polypeptides or anti-Zalpha12 antibodies, or bioactive fragments or portions thereof, can be coupled to detectable or cytotoxic molecules and delivered to a mammal having cells, tissues or organs that express the anti-complementary molecule.

Suitable detectable molecules may be directly or indirectly attached to the polypeptide or antibody, and

include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like. Suitable cytotoxic molecules may be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188 or yttrium-90 (either directly attached to the polypeptide or antibody, or indirectly attached through means of a chelating moiety, for instance). Polypeptides or antibodies may also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or cytotoxic molecule, the detectable or cytotoxic molecule can be conjugated with a member of a complementary/ anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes, biotin/streptavidin is an exemplary complementary/ anticomplementary pair.

In another embodiment, polypeptide-toxin fusion proteins or antibody-toxin fusion proteins can be used for targeted cell or tissue inhibition or ablation (for instance, to treat cancer cells or tissues). Alternatively, if the polypeptide has multiple functional domains (i.e., an activation domain or a ligand binding domain, plus a targeting domain), a fusion protein including only the targeting domain may be suitable for directing a detectable molecule, a cytotoxic molecule or a complementary molecule to a cell or tissue type of interest. In instances where the domain only fusion protein includes a complementary molecule, the anti-complementary molecule can be conjugated to a detectable or cytotoxic molecule. Such domain-complementary molecule fusion proteins thus represent a generic targeting vehicle for cell/tissue-specific delivery of generic anti-complementary-detectable/ cytotoxic molecule conjugates.

In another embodiment, Zalpha12-cytokine fusion proteins or antibody-cytokine fusion proteins can be used for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers), if the Zalpha12 polypeptide or anti-Zalpha12 antibody targets the hyperproliferative blood or bone marrow cell. See, generally, Hornick *et al.*, *Blood 89*:4437 (1997). The described fusion proteins enable targeting of a cytokine to a desired site of action, thereby providing an elevated local concentration of cytokine. Suitable Zalpha12 polypeptides or anti-Zalpha12 antibodies target an undesirable cell or tissue (*i.e.*, a tumor or a leukemia), and the fused cytokine mediated improved target cell lysis by effector cells. Suitable cytokines for this purpose include interleukin 2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), for instance.

In yet another embodiment, if the Zalpha12 polypeptide or anti-Zalpha12 antibody targets vascular cells or tissues, such polypeptide or antibody may be conjugated with a radionuclide, and particularly with a beta-emitting radionuclide, to reduce restenosis. Such therapeutic approach poses less danger to clinicians who administer the radioactive therapy. For instance, iridium-192 impregnated ribbons placed into stented vessels of patients until the required radiation dose was delivered showed decreased tissue growth in the vessel and greater luminal diameter than the control group, which received placebo ribbons. Further, revascularisation and stent thrombosis were significantly lower in the treatment group. Similar results are predicted with targeting of a bioactive conjugate containing a radionuclide, as described herein.

The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

USES OF POLYNUCLEOTIDE/POLYPEPTIDE:

Molecules of the present invention can be used to identify and isolate receptors involved in spermatogenesis, steroidogenesis, testicular differentiation and regulatory control of the hypothalamic-pituitary-gonadal axis or receptors of the immune system. For example, proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column, *Immobilized Affinity Ligand Techniques*, Hermanson *et al.*, eds., pp.195-202 (Academic Press, San Diego, CA, 1992,). Proteins and peptides can also be radiolabeled, *Methods in Enzymol.*, vol. 182, "Guide to Protein Purification", M. Deutscher, ed., pp 721-737 (Acad. Press, San Diego, 1990) or photoaffinity labeled, Brunner *et al.*, *Ann. Rev. Biochem.* *62*:483-514 (1993) and Fedan *et al.*, *Biochem. Pharmacol.* *33*:1167 (1984) and specific cell-surface proteins can be identified.

The molecules of the present invention will be useful for testing disorders of the reproductive system and immunological systems.

GENE THERAPY:

Polynucleotides encoding Zalpha12 polypeptides are useful within gene therapy applications where it is desired to increase or inhibit Zalpha12 activity. If a mammal has a mutated or absent Zalpha12 gene, the Zalpha12 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a Zalpha12 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective

viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 (HSV1) vector, Kaplitt *et al.*, *Molec. Cell. Neurosci.* *2*:320 (1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.*, *J. Clin. Invest.* *90*:626 (1992); and a defective adeno-associated virus vector, Samulski *et al.*, *J. Virol.* *61*:3096 (1987); Samulski *et al.*, *J. Virol.* *63*:3822 (1989).

In another embodiment, a Zalpha12 gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.* *Cell* *33*:153, 1983; Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, *J. Virol.* *62*:1120 (1988); Temin *et al.*, U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995 by Dougherty *et al.*; and Kuo *et al.*, *Blood* *82*:845 (1993). Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker, Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:7413 (1987); Mackey *et al.*, *Proc. Natl. Acad. Sci. USA* *85*:8027 (1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For

instance, directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, *e.g.*, Wu *et al.*, *J. Biol. Chem. 267*:963 (1992); Wu *et al.*, *J. Biol. Chem. 263*:14621-4, 1988.

Antisense methodology can be used to inhibit Zalpha12 gene transcription, such as to inhibit cell proliferation in vivo. Polynucleotides that are complementary to a segment of a Zalpha12-encoding polynucleotide (*e.g.*, a polynucleotide as set froth in SEQ ID NO:1) are designed to bind to Zalpha12-encoding mRNA and to inhibit translation of such mRNA. Such antisense polynucleotides are used to inhibit expression of Zalpha12 polypeptide-encoding genes in cell culture or in a subject.

The present invention also provides reagents which will find use in diagnostic applications. For example, the Zalpha12 gene, a probe comprising Zalpha12 DNA or RNA or a subsequence thereof can be used to determine if the Zalpha12 gene is present on chromosome 22q13.1 or if a mutation has occurred. Detectable chromosomal aberrations at the Zalpha12 gene locus

include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Such aberrations can be detected using polynucleotides of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, and other genetic linkage analysis techniques known in the art (Sambrook *et al.*, *ibid.*; Ausubel et. al., *ibid.*; Marian, *Chest 108*:255 (1995).

Transgenic mice, engineered to express the Zalpha12 gene, and mice that exhibit a complete absence of Zalpha12 gene function, referred to as "knockout mice", Snouwaert *et al.*, *Science 257*:1083 (1992), may also be generated, Lowell *et al.*, *Nature 366*:740-42 (1993). These mice may be employed to study the Zalpha12 gene and the protein encoded thereby in an *in vivo* system.

CHROMOSOMAL LOCALIZATION:

Zalpha12 has been mapped to chromosome 22q13.1. Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox *et al.*, *Science 250*:245 (1990). Partial or full knowledge of a gene's sequence allows one to design PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Radiation hybrid mapping panels are commercially available which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL). These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered

genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful for a number of purposes, including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

Sequence tagged sites (STSs) can also be used independently for chromosomal localization. An STS is a DNA sequence that is unique in the human genome and can be used as a reference point for a particular chromosome or region of a chromosome. An STS is defined by a pair of oligonucleotide primers that are used in a polymerase chain reaction to specifically detect this site in the presence of all other genomic sequences. Since STSs are based solely on DNA sequence they can be completely described within an electronic database, for example, Database of Sequence Tagged Sites (dbSTS), GenBank, (National Center for Biological Information, National Institutes of Health, Bethesda, MD http://www.ncbi.nlm.nih.gov), and can be searched with a gene sequence of interest for the mapping data contained within these short genomic landmark STS sequences.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a Zalpha12 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one

or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: *The Science and Practice of Pharmacy*, Gennaro, ed.,(Mack Publishing Co., Easton, PA, 19th ed., 1995). Therapeutic doses will generally be in the range of 0.1 to 100 µg/kg of patient weight per day, preferably 0.5-20 mg/kg per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years. Administration of the protein can be subcutaneous, intraperitoneal or rectal depending on the disease to be treated.

Tissue Expression and Use

Zalpha12 represents a novel polypeptide with a putative signal peptide leader sequence and alpha helical structure. Several putative isoforms have been identified.. Therefore this gene may encode a secreted polypeptide with secondary structure indicating it is a member of the four helix bundle cytokine family. Alternatively, this polypeptide may have other activities associated with other biological functions including: enzymatic activity, association with the cell membrane, or function as a carrier protein.

Northern blot analysis detects transcripts for zalpha12 in spleen, thymus, testis, small intestine, colon, PBL, stomach, lymph node, trachea, and bone marrow. Many of these organs have important immunological function or contain cells which play a role in the immune system.

Zalpha12 is also expressed in CD4+ enriched peripheral T cells and to a lesser degree in CD8+ T cells. There is very weak expression in CD19+ B cells suggesting that Zalpha12 has expression in T lymphoid lineage cells and little if any in B lymphoid lineage cells. There is elevated expression of zalpha12 in RNA derived from a 7 day human mixed lymphocyte reaction. This suggests that zalpha12 expression is regulated and increases after T cell activation.

Use of Zalpha12

Zalpha12 can be administered to an immunocompromised mammal, preferably a human, such as cancer patients who have undergone chemotherapy, AIDS patients and the elderly. This will stimulate their immune systems. Zalpha12 can also be used as a vaccine adjuvant to be administered before, with or after the administration of a vaccine. Zalpha12 may also be administered to stimulate the immune system to attack tumors.

Use of Antagonists of Zalpha12

An antagonist to Zalpha12, such as an antibody, soluble receptor or small molecule antagonist can be administered to a mammal, preferably a human, to alleviate an inflammatory response. Antagonists, such as antibodies, to Zalpha12 can be used to treat patients having inflammatory related diseases such arteriosclerotic heart disease [see Paulsson,G. *et al.*, *Arterioscler Thromb. Vasc. Biol.*, *20*:10-17 (2000)], inflammatory bowel disease, Crohn's disease, rheumatoid arthritis and pancreatitis.

The invention is further illustrated by the following non-limiting examples.

Example 1

Cloning of Zalpha12

Zalpha12 was discovered by using the Expressed Sequence Tag of SEQ ID NO:7 as a probe in a spleen cDNA library. The library was constructed using 1 microgram

(μg) of polyA RNA isolated from the spleen tissue of a 2-year-old Hispanic male, who died from cerebral anoxia. cDNA synthesis was initiated using a NotI-oligo(dT) primer. Double-stranded cDNA was blunted, ligated to EcoRI adaptors, digested with NotI, size-selected, and cloned into the NotI and EcoRI sites of a vector. The sequenced clone resulted in the full-length DNA and polypeptide sequences of SEQ ID NOs: 1 and 2.

The EST of SEQ ID NO: 17 resulted in the discovery of a second clone. The clone was sequence and found to have an unspliced intron. Oligonucleotide primers SEQ ID NOs: 11 and 12 were designed to amplify the full-length sequence without the intron sequence. The PCR was performed using lymph node MARATHON READY® cDNA (Clontech, Palo Alto, CA) as template. An 823 bp was cut out and gel purified using the QIAQUICK® gel extraction kit (Qiagen, Santa Clarita, CA). The excised PCR fragment was sequenced and did not contain the intron sequence. The next step was to see if the clone of SEQ ID NO:17 had another initiation codon upstream. A RACE® reaction using lymph node cDNA was performed to obtain 5' sequence. Oligonucleotide primers SEQ ID NOs:13 and 14 were designed for 5' RACE® reactions and nested RACE®. The RACE® reactions revealed an additional initiation codon upstream. The actual full-length was obtained through PCR using primers SEQ ID NOs:15 and 16 which flanked the coding sequence. Six bands were amplified and each was gel purified using the QIAQUICK® gel extraction kit and subcloned into plasmid pCR2.1TOPO vector using the TOPO TA® cloning kit (Invitrogen, Carlsbad, CA). The clones were sequenced and resulted in the full-length sequences of SEQ ID NOs: 3, 4, 5 and 6.

Thus, three variants were discovered, namely SEQ ID NO:1 and 2, SEQ ID NO:3 and 4, and SEQ ID NOs: 5 and 6.

Example 2

Northern Blot Analysis of *Zalpha12*

A northern blot analysis was carried out by standard techniques using the polynucleotides of SEQ ID NOs: 1 and 3. The results indicate that Zalpha12 is expressed in lymph nodes, activated mixed lymphocytes, spleen, thymus, testis, small intestines, human aortic endothelial cells, smooth muscle, kidney, mast cells, eosinophils, tonsils, pancreas, colon, peripheral blood lymphocytes (PBL), stomach, trachea, T-cells including $CD4^+$ and $CD8^+$ cells, and bone marrow.

Zalpha12 is also expressed by the following cell lines: HL60 (ATCC 45500), an acute promyelocytic leukemia cell line; Jurkat cells (ATCC TIB-152) a T lymphocyte from acute T cell leukemia; MOLT-4 cells (ATCC CRL-1582)a T lymphoblast from acute lymphoblastic leukemia; and HuT 78 cells (ATCC TIB-161) a cutaneous T lymphocyte from a lymphoma.

From these data, it can be concluded that Zalpha12 is a cytokine involved in the inflammation cascade. Antagonists of Zalpha12 can be used to alleviate inflammation related to a number of diseases such as arteriosclerotic heart disease, cardiovascular disease, rheumatoid arthritis, inflammatory bowel disease, and Crohn's disease.

Example 3

Zalpha12 Anti-peptide Antibodies

Polyclonal anti-peptide antibodies were prepared by immunizing two female New Zealand white rabbits with the peptide huzalpha12X1-1 AQQHKGSLQKDPLLSQACVGCLEALLDYLDAR (SEQ ID NO:41) or the peptide from SEQ ID NO:4 PLPATKDTVLAPLRMSQVRSLVIGLQNLLVC (SEQ ID NO:42) or the peptide from SEQ ID NO:4 CEGLPPSTSSGQPPLQDMLCLGGVAVSLSHIRN (SEQ ID NO:43) or the

peptide from SEQ ID NO:4 FMRYRSSSVLSHEEC (SEQ ID NO:44). The peptides were synthesized using an Applied Biosystems Model 431A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) according to manufacturer's instructions. The peptides were then conjugated to the carrier protein maleimide-activated keyhole limpet hemocyanin (KLH) through cysteine residues according to manufacturer's instructions. (Pierce, Rockford, IL). The rabbits were each given an initial intraperitoneal (IP) injection of 200 µg of the conjugated peptide in Complete Freund's Adjuvant (Pierce, Rockford, IL) followed by booster IP injections of 100 µg conjugated peptide in Incomplete Freund's Adjuvant every three weeks. Seven to ten days after the administration of the third booster injection, the animals were bled and the serum was collected. The rabbits were then boosted and bled every three weeks.

The zalpha12 peptide-specific Rabbit seras were characterized by an ELISA titer check using 1 µg/ml of the peptide used to make the antibody as an antibody target. The 2 rabbit seras to the SEQ ID NO:41 peptide have titer to their specific peptide at a dilution of 1:5E5 (1:100000). The 2 rabbit seras to the SEQ ID NO:42 peptide had titer to their specific peptide at a dilution of 1:5E4 (1:10,000). The 2 rabbit seras to the SEQ ID NO:43 peptide had titer to their specific peptide at a dilution of 1:5E4. The 2 rabbit seras to the SEQ ID NO:44 peptide had titer to their specific peptide at a dilution of 1:5E5.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs.2, 4, 6, 8, 9, 10, 18, 19, 20, 25, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39, 40, 41, 42, 43 and 44 or a polypeptide which is at least 80% identical to said polypeptide.

2. An isolated polypeptide comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs.2, 4, 6, 8, 9, 10 or a polypeptide which is at least 70% identical to said polypeptide.

3. An isolated polynucleotide which encodes a polypeptide, wherein said polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9, 10, 18, 19, 20, 25, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39 and 40 or a polypeptide which is at least 80% identical to said polypeptide.

4. An isolated polynucleotide which encodes a polypeptide, wherein said polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10 or a polypeptide which is at least 70% identical to said polypeptide.

5. An antibody which specifically binds to a polypeptide, wherein said polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9, 10, 18, 19, 20, 25, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39 and 40.

6. An antibody of claim 5 which specifically binds to a polypeptide, wherein said polypeptide is comprised of an

amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9, and 10.

7. An anti-idiotypic antibody which specifically binds to an antibody which specifically binds to a polypeptide, wherein said polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9, 10, 18, 19, 20, 25, 26, 27, 28, 29, 30, 35, 36, 37, 38, 39 and 40.

8. An anti-idiotypic antibody of claim 7 wherein the polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10.

9. The use of an antagonist to a polypeptide for treating inflammation wherein the polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10.

10. The use of claim 9 wherein the antagonist is an antibody which binds to a polypeptide comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10.

11. The use of an antagonist to a polypeptide for manufacturing a medicament for treating inflammation wherein the polypeptide is comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10.

12. The use of claim 11 wherein the antagonist is an antibody which binds to a polypeptide comprised of an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4, 6, 8, 9 and 10.

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Ala Leu Thr Leu Ala Lys Ala Asp Ser Pro Arg Thr Ala Leu Leu Cys
     10                  15                  20

tct gcc tgg ctg ctc act gcc tcc ttc tct gcc cag cag cac aag ggc     150
Ser Ala Trp Leu Leu Thr Ala Ser Phe Ser Ala Gln Gln His Lys Gly
 25                  30                  35                  40

agt ttg cag gtt cac cag aca ctc tct gtg gaa atg gac caa gta ttg     198
Ser Leu Gln Val His Gln Thr Leu Ser Val Glu Met Asp Gln Val Leu
                 45                  50                  55

aag gct ctc agc ttt cca aag aaa aag gct gca cta ctc tca gct gcc     246
Lys Ala Leu Ser Phe Pro Lys Lys Lys Ala Ala Leu Leu Ser Ala Ala
             60                  65                  70

atc tta tgc ttc ctg cgg aca gcc ctg cga caa agc ttt tcc tct gcc     294
Ile Leu Cys Phe Leu Arg Thr Ala Leu Arg Gln Ser Phe Ser Ser Ala
         75                  80                  85

ctg gta gcc ctg gtg ccc tca ggg gcc cag cca ctg cca gcc acc aag     342
Leu Val Ala Leu Val Pro Ser Gly Ala Gln Pro Leu Pro Ala Thr Lys
     90                  95                 100

gac act gtc cta gct cca ctg cga atg tcg caa gtc cgg tcc ctg gtc     390
Asp Thr Val Leu Ala Pro Leu Arg Met Ser Gln Val Arg Ser Leu Val
105                 110                 115                 120

att ggg ctg cag aac ctc ctg gtg cag aag gac cct cta ttg tcc cag     438
Ile Gly Leu Gln Asn Leu Leu Val Gln Lys Asp Pro Leu Leu Ser Gln
                125                 130                 135

gcc tgt gtt ggc tgc ctg gag gcc ttg ctt gac tac ctg gat gcc cgg     486
Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg
            140                 145                 150

agc cca gac att gct ctc cac gtg gcc tcc cag cct tgg aat cgg ttt     534
Ser Pro Asp Ile Ala Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe
        155                 160                 165
```

```
ttg ctg ttt acc ctc ttg gat gct gga gag aat tcc ttc ctc aga cct       582
Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro
    170                 175                 180

gag att ttg agg ctc atg acc ctg ttt atg cgg tac cgg agt agc agt       630
Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr Arg Ser Ser Ser
185                 190                 195                 200

gtc ctc tct cat gaa gag gtg ggt gat gtt ctg caa ggt gtg gct ttg       678
Val Leu Ser His Glu Glu Val Gly Asp Val Leu Gln Gly Val Ala Leu
                205                 210                 215

gct gac ctg tct acc ctc tcg aac acc aca ctc cag gcc ctg cat ggc       726
Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala Leu His Gly
            220                 225                 230

ttc ttc cag cag ctc cag agc atg gga cac ctg gct gac cac agc atg       774
Phe Phe Gln Gln Leu Gln Ser Met Gly His Leu Ala Asp His Ser Met
        235                 240                 245

gcc cag acc ctg cag gcc tcc ttg gag ggc ctt ccc cct agc acc tcc       822
Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro Ser Thr Ser
    250                 255                 260

tca ggc cag cca ccc ctg cag gac atg ctc tgc ctg gga ggg gtg gct       870
Ser Gly Gln Pro Pro Leu Gln Asp Met Leu Cys Leu Gly Gly Val Ala
265                 270                 275                 280

gta tcc ctg tcc cac atc aga aac tgatc                                 899
Val Ser Leu Ser His Ile Arg Asn
                285
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<210> 4
<211> 288
<212> PRT
<213> Homo sapiens

<400> 4

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Met Glu Leu Leu Glu Lys Met Leu Ala Leu Thr Leu Ala Lys Ala Asp
 1               5                  10                  15
Ser Pro Arg Thr Ala Leu Leu Cys Ser Ala Trp Leu Leu Thr Ala Ser
            20                  25                  30
Phe Ser Ala Gln Gln His Lys Gly Ser Leu Gln Val His Gln Thr Leu
        35                  40                  45
```

```
Ser Val Glu Met Asp Gln Val Leu Lys Ala Leu Ser Phe Pro Lys Lys
    50                  55                  60
Lys Ala Ala Leu Leu Ser Ala Ala Ile Leu Cys Phe Leu Arg Thr Ala
65                  70                  75                  80
Leu Arg Gln Ser Phe Ser Ser Ala Leu Val Ala Leu Val Pro Ser Gly
                85                  90                  95
Ala Gln Pro Leu Pro Ala Thr Lys Asp Thr Val Leu Ala Pro Leu Arg
            100                 105                 110
Met Ser Gln Val Arg Ser Leu Val Ile Gly Leu Gln Asn Leu Leu Val
        115                 120                 125
Gln Lys Asp Pro Leu Leu Ser Gln Ala Cys Val Gly Cys Leu Glu Ala
    130                 135                 140
Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala Leu His Val
145                 150                 155                 160
Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu Leu Asp Ala
                165                 170                 175
Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu
            180                 185                 190
Phe Met Arg Tyr Arg Ser Ser Ser Val Leu Ser His Glu Glu Val Gly
        195                 200                 205
Asp Val Leu Gln Gly Val Ala Leu Ala Asp Leu Ser Thr Leu Ser Asn
    210                 215                 220
Thr Thr Leu Gln Ala Leu His Gly Phe Phe Gln Gln Leu Gln Ser Met
225                 230                 235                 240
Gly His Leu Ala Asp His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu
                245                 250                 255
Glu Gly Leu Pro Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln Asp
            260                 265                 270
Met Leu Cys Leu Gly Gly Val Ala Val Ser Leu Ser His Ile Arg Asn
        275                 280                 285
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<210> 5
<211> 510
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (32)...(505)

<400> 5

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tgctgcagtg ctcctgagca gcacaggcct g atg gag ctt ctg gag aag atg      52
                                   Met Glu Leu Leu Glu Lys Met
                                    1               5
```

```
ctg gcc ctc acc ttg gca aag gca gat tct ccc agg act gca ctc ctc      100
Leu Ala Leu Thr Leu Ala Lys Ala Asp Ser Pro Arg Thr Ala Leu Leu
        10                  15                  20

tgc tct gcc tgg ctg ctc act gcc tcc ttc tct gcc cag cag cac aag      148
Cys Ser Ala Trp Leu Leu Thr Ala Ser Phe Ser Ala Gln Gln His Lys
    25                  30                  35

ggc agt ttg cag aag gac cct cta ttg tcc cag gcc tgt gtt ggc tgc      196
Gly Ser Leu Gln Lys Asp Pro Leu Leu Ser Gln Ala Cys Val Gly Cys
40                  45                  50                  55

ctg gag gcc ttg ctt gac tac ctg gat gcc cgg agc cca gac att gct      244
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala
                60                  65                  70

ctc cac gtg gcc tcc cag cct tgg aat cgg ttt ttg ctg ttt acc ctc      292
Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu
            75                  80                  85

ttg gat gct gga gag aat tcc ttc ctc aga cct gag att ttg agg ctc      340
Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu
        90                  95                  100

atg acc ctg ctc cag agc atg gga cac ctg gct gac cac agc atg gcc      388
Met Thr Leu Leu Gln Ser Met Gly His Leu Ala Asp His Ser Met Ala
    105                 110                 115

cag acc ctg cag gcc tcc ttg gag ggc ctt ccc cct agc acc tcc tca      436
Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro Ser Thr Ser Ser
120                 125                 130                 135

ggc cag cca ccc ctg cag gac atg ctc tgc ctg gga ggg gtg gct gta      484
Gly Gln Pro Pro Leu Gln Asp Met Leu Cys Leu Gly Gly Val Ala Val
                140                 145                 150

tcc ctg tcc cac atc aga aac tgatc                                    510
Ser Leu Ser His Ile Arg Asn
            155
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<210> 6
<211> 158
<212> PRT
<213> Homo sapiens

<400> 6

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Met Glu Leu Leu Glu Lys Met Leu Ala Leu Thr Leu Ala Lys Ala Asp
1               5                   10                  15
Ser Pro Arg Thr Ala Leu Leu Cys Ser Ala Trp Leu Leu Thr Ala Ser
            20                  25                  30
Phe Ser Ala Gln Gln His Lys Gly Ser Leu Gln Lys Asp Pro Leu Leu
        35                  40                  45
Ser Gln Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp
    50                  55                  60
Ala Arg Ser Pro Asp Ile Ala Leu His Val Ala Ser Gln Pro Trp Asn
65                  70                  75                  80
Arg Phe Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu Asn Ser Phe Leu
                85                  90                  95
Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Leu Gln Ser Met Gly His
            100                 105                 110
Leu Ala Asp His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly
        115                 120                 125
Leu Pro Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln Asp Met Leu
    130                 135                 140
Cys Leu Gly Gly Val Ala Val Ser Leu Ser His Ile Arg Asn
145                 150                 155
```

<210> 7
<211> 522
<212> DNA
<213> Homo sapiens

<400> 7

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catcagacca cacccagcag tcagaaaaga ggtgcagggg cccgggctgg gacagtgaag    60
agtgctgggc agtctgtggt cctctgtatc tcaacttttt catcttaaaa aaacaaatag   120
ggttgtgtgt gtggctggtg gtcataaggt cctttctggc tctaataacc tgagcttctg   180
ttatgaagct gggaccctta gagcctcagg atgatcctct gtttgtttgt gaaccccaat   240
caggtgctaa gcacatagtg gcacttagct gaagctcctc tgtaactcct gtgggccctg   300
cattgcccac ccccgacagc tgctgcagtg ctcctgagca gcacaggcct gatggagctt   360
ctggagaaga tgctggccct caccttggca aaggcagatt ctcccaggac tgcactcctc   420
tgctctgcct ggctgctcac tgcctccttc tctgcccagc agcacaaggg cagtttgcag   480
aaggaccctc tattgtccca ggcctgtgtt ggctgcctgg ag                      522
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<210> 8
<211> 168
<212> PRT
<213> Homo sapiens

<400> 8

Ala Gln Gln His Lys Gly Ser Leu Gln Lys Asp Pro Leu Leu Ser Gln
1 5 10 15
Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg
20 25 30
Ser Pro Asp Ile Ala Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe
35 40 45
Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro
50 55 60
Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr Arg Ser Ser Ser
65 70 75 80
Val Leu Ser His Glu Glu Val Gly Asp Val Leu Gln Gly Val Ala Leu
85 90 95
Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala Leu His Gly
100 105 110
Phe Phe Gln Gln Leu Gln Ser Met Gly His Leu Ala Asp His Ser Met
115 120 125
Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro Ser Thr Ser
130 135 140
Ser Gly Gln Pro Pro Leu Gln Asp Met Leu Cys Leu Gly Gly Val Ala
145 150 155 160
Val Ser Leu Ser His Ile Arg Asn
165

<210> 9
<211> 254
<212> PRT
<213> Homo sapiens

<400> 9
Ala Gln Gln His Lys Gly Ser Leu Gln Val His Gln Thr Leu Ser Val
1 5 10 15
Glu Met Asp Gln Val Leu Lys Ala Leu Ser Phe Pro Lys Lys Lys Ala
20 25 30
Ala Leu Leu Ser Ala Ala Ile Leu Cys Phe Leu Arg Thr Ala Leu Arg
35 40 45
Gln Ser Phe Ser Ser Ala Leu Val Ala Leu Val Pro Ser Gly Ala Gln
50 55 60
Pro Leu Pro Ala Thr Lys Asp Thr Val Leu Ala Pro Leu Arg Met Ser
65 70 75 80
Gln Val Arg Ser Leu Val Ile Gly Leu Gln Asn Leu Leu Val Gln Lys
85 90 95
Asp Pro Leu Leu Ser Gln Ala Cys Val Gly Cys Leu Glu Ala Leu Leu
100 105 110
Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala Leu His Val Ala Ser
115 120 125

Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu
130 135 140
Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met
145 150 155 160
Arg Tyr Arg Ser Ser Ser Val Leu Ser His Glu Glu Val Gly Asp Val
165 170 175
Leu Gln Gly Val Ala Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr
180 185 190
Leu Gln Ala Leu His Gly Phe Phe Gln Gln Leu Gln Ser Met Gly His
195 200 205
Leu Ala Asp His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly
210 215 220
Leu Pro Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln Asp Met Leu
225 230 235 240
Cys Leu Gly Gly Val Ala Val Ser Leu Ser His Ile Arg Asn
245 250

<210> 10
<211> 124
<212> PRT
<213> Homo sapiens

<400> 10
Ala Gln Gln His Lys Gly Ser Leu Gln Lys Asp Pro Leu Leu Ser Gln
1 5 10 15
Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg
20 25 30
Ser Pro Asp Ile Ala Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe
35 40 45
Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro
50 55 60
Glu Ile Leu Arg Leu Met Thr Leu Leu Gln Ser Met Gly His Leu Ala
65 70 75 80
Asp His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro
85 90 95
Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln Asp Met Leu Cys Leu
100 105 110
Gly Gly Val Ala Val Ser Leu Ser His Ile Arg Asn
115 120

<210> 11
<211> 19
<212> DNA
<213> Homo sapiens

<400> 11
agtttgcagg ttcaccaga 19

<210> 12
<211> 20
<212> DNA
<213> Homo sapiens

<400> 12
caattatgcc ctttgtctcc 20

<210> 13
<211> 24
<212> DNA
<213> Homo sapiens

<400> 13
ccagggctac cagggcagag gaaa 24

<210> 14
<211> 24
<212> DNA
<213> Homo sapiens

<400> 14
ccgcaggaag cataagatgg cagc 24

<210> 15
<211> 23
<212> DNA
<213> Homo sapiens

<400> 15
cttctgggcc ttcaagtcct gag 23

<210> 16
<211> 20
<212> DNA
<213> Homo sapiens

<400> 16
ccttgcccac ccccgacagc 20

<210> 17
<211> 197

<212> DNA
<213> Homo sapiens

<400> 17
gcagtttgca ggttcaccag gcactctctg tggaaatgga ccaagtattg aaggctctca 60
gctttccaaa gaaaaaggct gcactactct cagctgccat cttatgcttc ctgcggacag 120
cccttcgaca aagcttttcc tctgccctgg tagccctggt gccctcaggg gcccagccac 180
tgccagccac caaggac 197

<210> 18
<211> 71
<212> PRT
<213> Homo sapiens

<400> 18
Gln Gln His Lys Gly Ser Leu Gln Val His Gln Thr Leu Ser Val Glu
1 5 10 15
Met Asp Gln Val Leu Lys Ala Leu Ser Phe Pro Lys Lys Lys Ala Ala
20 25 30
Leu Leu Ser Ala Ala Ile Leu Cys Phe Leu Arg Thr Ala Leu Arg Gln
35 40 45
Ser Phe Ser Ser Ala Leu Val Ala Leu Val Pro Ser Gly Ala Gln Pro
50 55 60
Leu Pro Ala Thr Lys Asp Thr
65 70

<210> 19
<211> 69
<212> PRT
<213> Homo sapiens

<400> 19
Gln Lys Asp Pro Leu Leu Ser Gln Ala Cys Val Gly Cys Leu Glu Ala
1 5 10 15
Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala Leu His Val
20 25 30
Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu Leu Asp Ala
35 40 45
Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu
50 55 60
Phe Met Arg Tyr Arg
65

<210> 20
<211> 76

<212> PRT
<213> Homo sapiens

<400> 20
Arg Ser Ser Ser Val Leu Ser His Glu Glu Val Gly Asp Val Leu Gln
1   5   10   15
Gly Val Ala Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln
            20   25   30
Ala Leu His Gly Phe Phe Gln Gln Leu Gln Ser Met Gly His Leu Ala
        35   40   45
Asp His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro
    50   55   60
Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln Asp
65   70   75

<210> 21
<211> 15
<212> PRT
<213> Homo sapiens

<400> 21
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile
1   5   10   15

<210> 22
<211> 15
<212> PRT
<213> Homo sapiens

<400> 22
Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr
1   5   10   15

<210> 23
<211> 15
<212> PRT
<213> Homo sapiens

<400> 23
Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala Leu
1   5   10   15

<210> 24
<211> 15
<212> PRT

<213> Homo sapiens

<400> 24
Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro Ser
1 5 10 15

<210> 25
<211> 55
<212> PRT
<213> Homo sapiens

<400> 25
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala
1 5 10 15
Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu
20 25 30
Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu
35 40 45
Met Thr Leu Phe Met Arg Tyr
50 55

<210> 26
<211> 89
<212> PRT
<213> Homo sapiens

<400> 26
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala
1 5 10 15
Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu
20 25 30
Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu
35 40 45
Met Thr Leu Phe Met Arg Tyr Arg Ser Ser Ser Val Leu Ser His Glu
50 55 60
Glu Val Gly Asp Val Leu Gln Gly Val Ala Leu Ala Asp Leu Ser Thr
65 70 75 80
Leu Ser Asn Thr Thr Leu Gln Ala Leu
85

<210> 27
<211> 121
<212> PRT
<213> Homo sapiens

<400> 27

Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala
1 5 10 15
Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu
20 25 30
Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu
35 40 45
Met Thr Leu Phe Met Arg Tyr Arg Ser Ser Ser Val Leu Ser His Glu
50 55 60
Glu Val Gly Asp Val Leu Gln Gly Val Ala Leu Ala Asp Leu Ser Thr
65 70 75 80
Leu Ser Asn Thr Thr Leu Gln Ala Leu His Gly Phe Phe Gln Gln Leu
85 90 95
Gln Ser Met Gly His Leu Ala Asp His Ser Met Ala Gln Thr Leu Gln
100 105 110
Ala Ser Leu Glu Gly Leu Pro Pro Ser
115 120

<210> 28
<211> 81
<212> PRT
<213> Homo sapiens

<400> 28

Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr Arg
1 5 10 15
Ser Ser Ser Val Leu Ser His Glu Glu Val Gly Asp Val Leu Gln Gly
20 25 30
Val Ala Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala
35 40 45
Leu His Gly Phe Phe Gln Gln Leu Gln Ser Met Gly His Leu Ala Asp
50 55 60
His Ser Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro
65 70 75 80
Ser

<210> 29
<211> 49
<212> PRT
<213> Homo sapiens

<400> 29

Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr Arg
1 5 10 15

Ser Ser Ser Val Leu Ser His Glu Glu Val Gly Asp Val Leu Gln Gly
20 25 30
Val Ala Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala
35 40 45
Leu

<210> 30
<211> 47
<212> PRT
<213> Homo sapiens

<400> 30
Leu Ala Asp Leu Ser Thr Leu Ser Asn Thr Thr Leu Gln Ala Leu His
1 5 10 15
Gly Phe Phe Gln Gln Leu Gln Ser Met Gly His Leu Ala Asp His Ser
20 25 30
Met Ala Gln Thr Leu Gln Ala Ser Leu Glu Gly Leu Pro Pro Ser
35 40 45

<210> 31
<211> 15
<212> PRT
<213> Homo sapiens

<400> 31
His Gln Thr Leu Ser Val Glu Met Asp Gln Val Leu Lys Ala Leu
1 5 10 15

<210> 32
<211> 15
<212> PRT
<213> Homo sapiens

<400> 32
Val Arg Ser Leu Val Ile Gly Leu Gln Asn Leu Leu Val Gln Lys
1 5 10 15

<210> 33
<211> 15
<212> PRT
<213> Homo sapiens

<400> 33
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile

1 5 10 15

<210> 34
<211> 15
<212> PRT
<213> Homo sapiens

<400> 34
Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg Tyr
1 5 10 15

<210> 35
<211> 86
<212> PRT
<213> Homo sapiens

<400> 35
His Gln Thr Leu Ser Val Glu Met Asp Gln Val Leu Lys Ala Leu Ser
1 5 10 15
Phe Pro Lys Lys Lys Ala Ala Leu Leu Ser Ala Ala Ile Leu Cys Phe
20 25 30
Leu Arg Thr Ala Leu Arg Gln Ser Phe Ser Ser Ala Leu Val Ala Leu
35 40 45
Val Pro Ser Gly Ala Gln Pro Leu Pro Ala Thr Lys Asp Thr Val Leu
50 55 60
Ala Pro Leu Arg Met Ser Gln Val Arg Ser Leu Val Ile Gly Leu Gln
65 70 75 80
Asn Leu Leu Val Gln Lys
85

<210> 36
<211> 112
<212> PRT
<213> Homo sapiens

<400> 36
His Gln Thr Leu Ser Val Glu Met Asp Gln Val Leu Lys Ala Leu Ser
1 5 10 15
Phe Pro Lys Lys Lys Ala Ala Leu Leu Ser Ala Ala Ile Leu Cys Phe
20 25 30
Leu Arg Thr Ala Leu Arg Gln Ser Phe Ser Ser Ala Leu Val Ala Leu
35 40 45
Val Pro Ser Gly Ala Gln Pro Leu Pro Ala Thr Lys Asp Thr Val Leu
50 55 60
Ala Pro Leu Arg Met Ser Gln Val Arg Ser Leu Val Ile Gly Leu Gln

65 70 75 80
Asn Leu Leu Val Gln Lys Asp Pro Leu Leu Ser Gln Ala Cys Val Gly
85 90 95
Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile
100 105 110

<210> 37
<211> 152
<212> PRT
<213> Homo sapiens

<400> 37
His Gln Thr Leu Ser Val Glu Met Asp Gln Val Leu Lys Ala Leu Ser
1 5 10 15
Phe Pro Lys Lys Lys Ala Ala Leu Leu Ser Ala Ala Ile Leu Cys Phe
20 25 30
Leu Arg Thr Ala Leu Arg Gln Ser Phe Ser Ser Ala Leu Val Ala Leu
35 40 45
Val Pro Ser Gly Ala Gln Pro Leu Pro Ala Thr Lys Asp Thr Val Leu
50 55 60
Ala Pro Leu Arg Met Ser Gln Val Arg Ser Leu Val Ile Gly Leu Gln
65 70 75 80
Asn Leu Leu Val Gln Lys Asp Pro Leu Leu Ser Gln Ala Cys Val Gly
85 90 95
Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile
100 105 110
Ala Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr
115 120 125
Leu Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg
130 135 140
Leu Met Thr Leu Phe Met Arg Tyr
145 150

<210> 38
<211> 81
<212> PRT
<213> Homo sapiens

<400> 38
Val Arg Ser Leu Val Ile Gly Leu Gln Asn Leu Leu Val Gln Lys Asp
1 5 10 15
Pro Leu Leu Ser Gln Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp
20 25 30
Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala Leu His Val Ala Ser Gln
35 40 45

Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu Leu Asp Ala Gly Glu Asn
    50   55   60
Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu Met Thr Leu Phe Met Arg
65   70   75   80
Tyr

<210> 39
<211> 41
<212> PRT
<213> Homo sapiens

<400> 39
Val Arg Ser Leu Val Ile Gly Leu Gln Asn Leu Leu Val Gln Lys Asp
 1   5   10   15
Pro Leu Leu Ser Gln Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp
            20   25   30
Tyr Leu Asp Ala Arg Ser Pro Asp Ile
        35   40

<210> 40
<211> 55
<212> PRT
<213> Homo sapiens

<400> 40
Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg Ser Pro Asp Ile Ala
 1   5   10   15
Leu His Val Ala Ser Gln Pro Trp Asn Arg Phe Leu Leu Phe Thr Leu
            20   25   30
Leu Asp Ala Gly Glu Asn Ser Phe Leu Arg Pro Glu Ile Leu Arg Leu
        35   40   45
Met Thr Leu Phe Met Arg Tyr
    50   55

<210> 41
<211> 32
<212> PRT
<213> Homo sapiens

<400> 41
Ala Gln Gln His Lys Gly Ser Leu Gln Lys Asp Pro Leu Leu Ser Gln
 1   5   10   15
Ala Cys Val Gly Cys Leu Glu Ala Leu Leu Asp Tyr Leu Asp Ala Arg
            20   25   30

<210> 42
<211> 31
<212> PRT
<213> Homo sapiens

<400> 42
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<400> 43
Cys Glu Gly Leu Pro Pro Ser Thr Ser Ser Gly Gln Pro Pro Leu Gln
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Asp Met Leu Cys Leu Gly Gly Val Ala Val Ser Leu Ser His Ile Arg
20 25 30
Asn

<210> 44
<211> 15
<212> PRT
<213> Homo sapiens

<400> 44
Phe Met Arg Tyr Arg Ser Ser Ser Val Leu Ser His Glu Glu Cys
1 5 10 15

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/01015

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/52 C12N15/19 C07K16/24 A61K38/19 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES,14 May 1997 (1997-05-14), XP002138914 HINXTON, GB AC = AA419437. zv01g08.r1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:746270 5', mRNA sequence. EST. ---	3,4
A	WO 95 13393 A (CELL THERAPEUTICS INC) 18 May 1995 (1995-05-18) the whole document ---	1
A	WO 93 21308 A (UNIV HOSPITAL) 28 October 1993 (1993-10-28) the whole document ---	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2000	14/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mateo Rosell, A.M.

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna   il Application No
PCT/US 00/01015

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 29720 A (ZYMOGENETICS INC) 17 June 1999 (1999-06-17) the whole document	1,6-12
P,X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES,1 May 1999 (1999-05-01), XP002138913 HINXTON, GB AC = 095505. TrEMBLrel. DJ821D11.1 (PUTATIVE PROTEIN) (FRAGMENT). From nt 1-51. abstract	1-4
P,X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES,30 November 1999 (1999-11-30), XP002138915 HINXTON, GB AC = AW195720. xn85b08.x1 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone IMAGE:2701239 3', mRNA sequence. EST. abstract	3,4
P,X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES,2 August 1999 (1999-08-02), XP002138916 HINXTON, GB AC = AI928166. wp11h06.x1 NCI_CGAP_Kid12 Homo sapiens cDNA clone IMAGE:2464571 3', mRNA sequence. EST. abstract	3,4

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INTERNATIONAL SEARCH REPORT	International application No. PCT/US 00/01015

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9,11

Present claims 9 and 11 relating to the use of an antagonist to the polypeptides comprised in the SEQ.ID.Nos. 2,4,6,8,9 and 10, could not be searched as its subject-matter was insuficiently disclosed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern   ıal Application No
PCT/US 00/01015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9513393 A	18-05-1995	AU 1091995 A EP 0738329 A	29-05-1995 23-10-1996
WO 9321308 A	28-10-1993	AU 4289193 A	18-11-1993
WO 9929720 A	17-06-1999	AU 1908599 A	28-06-1999